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Replication Rapidly Recovers and Continues in the Presence of Hydroxyurea in Escherichia coli

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Running title: Replication recovers and continues in hydroxyurea

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ABSTRACT

In both prokaryotes and eukaryotes, hydroxyurea is suggested to inhibit DNA replication by inactivating ribonucleotide reductase and depleting deoxyribonucleoside triphosphate pools. In this study, we show that the inhibition of replication in *Escherichia coli* is transient even at concentrations of 0.1 M hydroxyurea and that replication rapidly recovers and continues in its presence. The recovery of replication does not require the alternative ribonucleotide reductases, NrdEF and NrdDG, or translesion DNA polymerases, Pol II, Pol IV, or Pol V. Ribonucleotides are incorporated at higher frequencies during replication in the presence of hydroxyurea. However, these do not contribute significantly to the observed synthesis or toxicity. Hydroxyurea toxicity was only observed under conditions where the stability of hydroxyurea was compromised and byproducts, known to damage DNA directly, were allowed to accumulate. The results demonstrate that hydroxyurea is not a direct or specific inhibitor of DNA synthesis *in vivo*, and that the transient inhibition observed is most likely due to a general depletion of iron cofactors from enzymes when 0.1 M hydroxyurea is initially applied. Finally, the results support previous studies suggesting that hydroxyurea toxicity is mediated primarily through direct DNA damage induced by the breakdown products of hydroxyurea, rather than by inhibition of replication or depletion of deoxyribonucleotide levels in the cell.

IMPORTANCE:

Hydroxyurea is commonly suggested to function by inhibiting DNA replication through the inactivation of ribonucleotide reductase and depleting deoxyribonucleoside triphosphate pools. Here, we show that hydroxyurea only transiently inhibits replication in *Escherichia coli* before it rapidly recovers and continues in the presence of this drug. The recovery of replication does not depend on alternative ribonucleotide reductases, translesion synthesis, or RecA. Further we show that hydroxyurea toxicity is only observed after toxic intermediates that accumulate when hydroxyurea breaks down, damage DNA and induce lethality. The results demonstrate that hydroxyurea toxicity is mediated indirectly by the formation of DNA damage, rather than by an inhibition of replication or

depletion of deoxyribonucleotide levels in the cell.

INTRODUCTION

Hydroxyurea has been used extensively in clinical settings as a chemotherapeutic, as an anti-viral, and for the treatment of sickle cell anemia (1–4). However, the drug's mechanism of action remains unclear. The most common mechanism of action proposed for hydroxyurea is the inhibition of ribonucleotide reductase, leading to depleted deoxyribonucleoside triphosphate (dNTP) pools that prevent DNA replication in both prokaryotes and eukaryotes (5–10). However, other mechanisms of action have also been proposed and include a general inhibition of metabolism due to disruption of iron-dependent enzymes in the cell (11–13), and direct induction of DNA damage (14, 15).

The most widely accepted mechanism of action for hydroxyurea proposes that it targets and inactivates ribonucleotide reductase. *In vitro,* several studies have demonstrated that addition of hydroxyurea to purified enzyme or extracts, inactivates the ribonucleotide reductase activity in both prokaryotes and eukaryotes (6, 16, 17). However, whether this is the mechanism that operates *in vivo* is less clear. Some early studies observed that hydroxyurea reduced dNTP concentrations immediately after addition in *Escherichia coli* cultures (10, 18). However other studies found that hydroxyurea's effects on the cell were not specific to dNTP concentrations and that transcription, translation, and growth were also inhibited to varying degrees (19, 20). Similarly, some studies in eukaryotes have found that hydroxyurea depletes cellular concentrations of dNTPs (21, 22), whereas others found dNTP levels remained largely unaffected by the presence of hydroxyurea (23, 24). In *E. coli,* the primary ribonucleotide reductase, essential for aerobic growth, is a two subunit, iron-dependent, Class Ia enzyme encoded by *nrdA* and *nrdB*. The larger R1 subunit (NrdA) contains an allosteric ATP- and nucleotide-binding site as well as the active site required for catalysis of ribonucleotide reduction; whereas the smaller R2 subunit (NrdB) contains a di-iron center that is required for initiation of NrdA activity (25). *E. coli* also encodes two alternative ribonucleotide reductases – NrdEF, a manganese-dependent, Class Ib ribonucleotide reductase that is active when iron is limiting (26) and NrdDG, a Class III enzyme that is required for growth under anaerobic conditions (27).

Ribonucleotide reductase is required for the *de novo* synthesis of dNTPs through a two-step reaction sequence that depends on free radical chemistry to reduce the 2'-carbon of a ribonucleotide to its deoxyribonucleotide derivative (28). In *E. coli*, generation of a stable tyrosyl free radical in the R2 subunit occurs following conversion of the NrdB protein from its ferrous to ferric form by molecular oxygen. Radical transfer from NrdB to a cysteine residue on NrdA then initiates catalysis of the ribonucleotide reduction reaction when substrate ribonucleotide and the allosteric effector ATP is bound to the R1 subunit (25, 28). *In vitro*, hydroxyurea inactivates ribonucleotide reductase by

scavenging the iron-stabilized, tyrosyl free radical from the active site of the R2 subunit (6, 8).

Alternatively, inactivation of ribonucleotide reductase by hydroxyurea may simply reflect a broad effect that this drug has on enzymes containing catalytic metals. Recent studies have found that hydroxyurea alters Fe-S centers that act as cofactors for various cellular enzymes (11–13). Hydroxyurea can also form complexes with iron and copper directly, and may act as a weak chelator *in vivo* (29, 30). Many Fe-S center proteins catalyze redox reactions that are required to maintain normal metabolism in the cell, suggesting that hydroxyurea could inhibit replication through a generalized effect on proteins requiring catalytic metals, of which NrdAB is one. This type of explanation would be consistent with many studies where inhibition of several processes is observed, including replication, transcription, and translation, and require extremely high hydroxyurea concentrations of 0.1-0.2 M (9, 19, 20, 31).

A third proposed mechanism of action for hydroxyurea suggests its toxicity is mediated by direct induction of DNA damage. The stability of hydroxyurea in its aqueous form is compromised over time or in the presence of heat, and breaks down to form N-hydroxyurethan and carbamoyloxyurea as well as hydrogen cyanide, nitric oxide, and peroxide (15, 32). Incubating hydroxyurea at temperatures of 37ºC and above was found to generate nitrosourea, *o*-carbamoylhydroxylamine and carbamoyloxyurea (32); while stocks of hydroxyurea stored at 37ºC for several days were observed to contain nitric oxide and hydrogen cyanide (15). These agents can react with DNA, inducing adducts and base damage. Hydroxyurea has also been proposed to induce oxidative free radicals that damage DNA through interactions with iron or other metals (14, 15, 33). Hydroxyurea treatment in *E. coli* cells has been indirectly shown to induce the formation of superoxide, which can subsequently be converted to hydrogen peroxide and react with iron to produce DNA-damaging hydroxyl radicals (33). Additionally, *in vitro* studies have correlated the formation of 8-hydroxy-2'-deoxyguanosine lesions with the production of nitric oxide in aqueous solutions of hydroxyurea that also contain copper (14).

Mutations in several replication and repair genes have been shown to affect survival in the presence of hydroxyurea. Loss of translesion DNA polymerases IV and V has been reported to confer a modest resistance to hydroxyurea (34). Conversely, *recA* mutants and AP endonuclease mutants *xthA nfo* are reported to be hypersensitive to hydroxyurea (15, 35, 36). These phenotypes have been proposed to be produced by a mechanism of action that could either be consistent with one that inhibits replication or one that induces DNA damage.

Thus, to further characterize hydroxyurea's mechanism of action, we characterized its inhibitory effect on the rate of DNA synthesis directly in *E. coli*. We show that hydroxyurea only transiently

inhibits DNA synthesis *in vivo*, and that this brief inhibition occurs only when high (0.1M) concentrations of drug are used. Following inhibition, DNA synthesis recovers and is able to continue for several hours in the presence of the drug. Using *recA* mutants as an indicator for the ability to replicate in the presence of DNA damage, we show that hydroxyurea toxicity is only observed under conditions where the stability of hydroxyurea is compromised and toxic intermediates, known to damage DNA directly are allowed to accumulate.

RESULTS

DNA replication in *E. coli* is only transiently inhibited by acute exposure to hydroxyurea.

Hydroxyurea is commonly thought to inhibit ongoing DNA replication indirectly by targeting the Class Ia ribonucleotide reductase (*nrdAB* gene products) of *E. coli* and depleting the dNTP pools required for DNA synthesis to occur (5, 6, 10). However, how hydroxyurea affects the rate of DNA synthesis *in vivo* has never been characterized directly. To examine this, we monitored the rate of DNA synthesis following exposure to 1-, 10-, and 100-mM hydroxyurea by incubating [^{14}C]thymine-prelabeled cultures with [^{3}H]thymidine for 2 min at various times after drug addition. The rate of DNA synthesis (^{3}H incorporation/2 min) could then be determined relative to the total amount of DNA present (^{14}C incorporation) at specific times following treatment. In each case, we included a mock-treated control to directly compare exposed cultures to unexposed cultures and ensure that any changes in the observed rates were due to the treatment rather than culture density.

Based on the predicted mode of action, we expected that following the addition of hydroxyurea, DNA synthesis would be inhibited for the duration of the time course. Surprisingly, this is not what we observed. Following drug addition, an initial and rapid decrease in the rate of DNA synthesis that varied directly as a function of hydroxyurea dose was observed (Figure 1). The rate of synthesis initially decreased by <10%, ~70%, and >90%, in the presence of 1- ,10-, and 100-mM hydroxyurea, respectively. However in each case, DNA synthesis began to rapidly recover after 5 minutes in the presence of the drug, and replication was fully restored within the 60-minute time course even in the presence of 100 mM hydroxyurea. The total DNA accumulation in each culture was reduced in a manner that correlated with the rate of DNA synthesis observed in each case. The average rate of DNA synthesis and total DNA accumulation was reduced by an average of 3% and 0%, 23% and 40%, 68% and 80% for 1-, 10-, and 100-mM hydroxyurea, respectively.

The recovery of DNA synthesis in the presence of hydroxyurea was unexpected given the proposed inhibitory activity of this drug on ribonucleotide reductase. The results indicate that the inhibition of replication by hydroxyurea requires high concentrations of the drug and that cells have

mechanisms that allow replication to rapidly resume in its presence.

The recovery of replication in the presence of hydroxyurea is not mediated by alternative ribonucleotide reductases or translesion DNA polymerases.

NrdAB encodes the primary ribonucleotide reductase that operates under aerobic conditions. However, *E. coli* encodes two alternative ribonucleotide reductases, *nrdE-F* and *nrdD-G* that each function under different growth conditions (26, 27), and are transcriptionally upregulated following hydroxyurea treatment (33). Thus, it is possible that these alternative ribonucleotide reductases, NrdEF or NrdDG, could be insensitive to hydroxyurea and compensate for NrdAB, thereby allowing replication to resume. To examine this directly, we constructed isogenic mutants lacking the cryptic Class Ib ribonucleotide reductase NrdEF, and the Class III ribonucleotide reductase-activating enzyme NrdG. The ability of these mutants to recover replication in the presence of 100 mM hydroxyurea was then compared to wild-type cells. As shown in Figure 2A, the rate of DNA synthesis decreased to the same extent and recovered with similar kinetics in wild-type, *nrdEF,* and *nrdG* cultures. These results indicate that the recovery of DNA synthesis in the presence of hydroxyurea does not depend on the alternative ribonucleotide reductases, NrdEF or NrdDG. However, we were unsuccessful in our attempts to construct an *nrdEF nrdDG* double mutant, perhaps suggesting a requirement for having at least one of these ribonucleotide reductases at some stage of growth on plates, in culture, or during strain construction.

We next considered the possibility that the recovery was mediated through the translesion DNA polymerases. *E. coli*'s three translesion DNA polymerase genes are transcriptionally upregulated in cultures challenged with hydroxyurea (33). Furthermore, Pol IV and Pol V mutants are reported to be more resistant to hydroxyurea than wild-type cells and the translesion DNA polymerases have been speculated to catalyze error-prone DNA synthesis when dNTP pools are low or unbalanced (34). However, when we examined the ability of mutants lacking all three translesion DNA polymerases (*polB dinB umuDC*) to recover replication in the presence of hydroxyurea, we observed no difference in recovery rates of these mutants compared to wild-type cells (Figure 2B). Similar to wild-type cells, the rate of DNA synthesis in hydroxyurea-treated *polB dinB umuDC* mutants decreased by more than 90% in the first 10 min following hydroxyurea addition, began to recover at 20 min and approached untreated levels by 50 min. The results demonstrate that the translesion DNA polymerases are not responsible for the replication occurring in the presence of hydroxyurea.

Ribonucleoside incorporation increases but does not account for the synthesis observed in the presence of hydroxyurea.

rNTP concentrations found in both eukaryotic and prokaryotic cells exceed that of dNTPs by 10- to 100-fold under normal growth conditions (37–39), leading to misincorporation of rNTPs into genomic DNA by polymerases at rates that have been estimated to be ~1 rNTP per 2.3 kb (38, 39). Treatment with hydroxyurea further increases rNDP levels and decreases dNTP pools by as much as 10-fold over untreated cells (10), raising the possibility that the synthesis occurring in the presence of hydroxyurea could contain significant amounts of ribonucleotides. In *E. coli*, single rNMP residues in genomic DNA are removed by the enzyme RNase HII (encoded by *rnhB*), while long RNA-DNA tracts like that found in Okazaki fragments are cleaved by RNase HI (encoded by *rnhA*) (40, 41).

To examine whether rNTP misincorporation might be contributing to the observed synthesis in the presence of hydroxyurea, we initially constructed isogenic mutants lacking *rnhB* and *rnhA*, and monitored overall DNA replication and rates of synthesis as described before. If the frequency of rNTP incorporation in DNA increases with hydroxyurea treatment, we predicted that in the absence of RNase HI or RNase HII these ribonucleotides would not be degraded or removed, potentially resulting in more [^{3}H]- and [^{14}C]-incorporation in these mutants relative to wild-type cells. Contrary to this prediction, we observed that the time and kinetics of replication resumption in *rnhB* cultures was similar to wild-type cells following hydroxyurea addition (Figure 3A). In *rnhA* mutants, the absence of RNase HI affected the rate, but not the time, at which DNA synthesis recovered after hydroxyurea addition. The overall rate of DNA synthesis was much reduced in hydroxyurea-treated *rnhA* cells compared to wild-type parent, however this difference could be attributed to *rnhA*'s slow growth phenotype and its role in Okazaki primer removal (41), and is reflected in the reduced DNA accumulation seen in mock-treated *rnhA* mutants (Figure 3A).

To determine if extensive ribonucleotide incorporation is occurring during growth in hydroxyurea, cultures of wild-type, *rnhA*, and *rnhB* cells were allowed to replicate in the presence or absence of 100 mM hydroxyurea for 4 hours, a period of time in which the DNA more than doubles (Figure 3B). Following this period, the genomic DNA was purified and incubated with 200 mM NaOH to hydrolyze the DNA backbone at rNMP moieties. The samples were then analyzed by denaturing alkali agarose gel electrophoresis. In wild-type cultures, genomic DNA purified and treated in this manner migrates with a size greater than 40kb (Figure 3B). Following 2 or 4 hours of replication in hydroxyurea, the DNA remains essentially intact and very little DNA fragmentation is observed following alkali denaturation, indicating that few ribonucleotides are present in the DNA. DNA similarly remains intact in *rnhA* mutants throughout the time course, suggesting that long stretches of

ribonucleotides are not incorporated or removed by RNase HI during hydroxyurea incubation. In contrast, significant ribonucleotide incorporation is observed in *rnhB* mutants. Although the genomic DNA of *rnhB* mutants is of high molecular weight when purified from cells before the addition of hydroxyurea, a significant loss of this high-molecular-weight DNA is observed at both 2 and 4 hours after incubation in hydroxyurea (Figure 3B). The loss of high-molecular-weight DNA is accompanied by the appearance of smaller fragments ranging in size between 4- and 40-kb. The observation demonstrates that during replication in hydroxyurea, elevated levels of ribonucleotides are incorporated and removed by RNase HII. The frequency of ribonucleotide incorporation in the presence of hydroxyurea remains below 1 ribonucleotide per several thousand bases, arguing that it is unlikely to account for the observed synthesis that occurs under these conditions.

Consistent with this last interpretation, we again monitored the overall DNA replication and rates of synthesis in wild-type cultures, but this time treated each of the lysed cell aliquots with 500 mM NaOH to remove any ribonucleotides prior to precipitation with trichloroacetic acid. Trichloroacetic acid effectively precipitates polynucleotides longer than 12 bp. We reasoned that if significant levels of ribonucleotides were incorporated during the recovery, the amount of precipitable nucleotides should be detectably reduced in the alkali-treated samples. However, as shown in Figure 3C, no difference was observed in the rate of recovery, or amount of DNA synthesis when alkali and non-alkali treated samples were compared. Thus, although the frequency of ribonucleotide incorporation increases during incubation in hydroxyurea, it cannot account for the synthesis occurring under these conditions.

Transient inhibition by hydroxyurea pauses, but does not disrupt replication, and recovery occurs independently of RecA.

To further characterize the nature of the transient replication inhibition seen after hydroxyurea addition, we examined whether the recovery of DNA synthesis depended upon RecA. Cells exposed to hydroxyurea upregulate *recA* gene expression as part of the SOS response (33, 42), and RecA is required for replication to recover following disruption by DNA damage (reviewed in (43)). To determine if the recovery of replication after hydroxyurea inhibition also required RecA, we examined the replication of *recA* cells in the presence of 100 mM hydroxyurea. As a control, and for the purpose of comparison, we also monitored the rate of replication in *recA* mutants following either UV irradiation or treatment with hydrogen peroxide. In both UV-irradiated or hydrogen peroxide-treated wild-type cultures, DNA synthesis was transiently inhibited before it was seen to recover (Figure 4A & B). The time that DNA synthesis recovers following UV or hydrogen peroxide has been shown to

correlate with the removal of the blocking lesions from the template through a process that is coupled with replication (44–49). By comparison, in *recA* mutants, no further DNA synthesis was observed after either UV irradiation or treatment with hydrogen peroxide, consistent with RecA's role in restoring replication after disruption by DNA damage (Figure 4A & B).

In contrast to UV irradiation or hydrogen peroxide, both wild-type and *recA* cells resumed DNA synthesis with similar kinetics following the addition of hydroxyurea (Figure 4C). DNA replication continued in the absence of RecA even when the time course was extended to six hours (Figure 4D). In both wild-type and *recA* mutant cultures, hydroxyurea treatment results in a modest, ~50%, reduction in DNA accumulation over this period. We interpret these results to imply that the transient inhibition caused by hydroxyurea stalls or pauses the replisome, but does not disrupt its integrity, as replication recovery occurs independently of RecA. This observation would also imply that no direct DNA damage is formed under these conditions, and is most consistent with the inhibitory effect of hydroxyurea resulting from a more global disruption of iron-dependent enzymes. Consistent with this interpretation, an inhibitory effect on transcription was also observed in the presence of hydroxyurea (Figure S1). In contrast to the case of DNA synthesis, no recovery of transcription was observed during the time course, suggesting that disruption of iron-dependent transcriptional enzymes is perhaps irreversible and requires new protein synthesis. Similarly, other studies have documented that several metabolic processes are inhibited in the presence of hydroxyurea (9, 19, 20, 31). Taken together, the observations further support the idea that HU is affecting a broad range of metabolic processes in the cell.

RecA contributes to survival and is required for the recovery of replication only under conditions when the stability of hydroxyurea is compromised and toxic intermediates accumulate.

The lack of a requirement for RecA to recover replication in the presence of hydroxyurea was unexpected as we and others observe that *recA* mutants exhibit reduced viability when spotted on agar plates containing hydroxyurea (Figure 5A and (36)). In surveying the literature, we noticed that the concentration of hydroxyurea required to reduce viability varies drastically between studies, and primarily appeared to depend on whether the assay was done on solid medium, such as agar plates, or in liquid culture. Whereas 5 to 10 mM was sufficient to reduce viability using hydroxyurea on agar plates, 100 to 200 mM concentrations are required when liquid cultures are used (15, 31, 33, 34, 36, 50). This discrepancy may arise due to the instability of hydroxyurea. Previous studies have found that hydroxyurea breaks down into toxic intermediates that include N-hydroxyurethan and carbamoyloxyurea, as well as hydrogen cyanide, nitric oxide, or peroxides over time or when exposed

to heat (14, 15, 32). Since both time and heat exposure are required to prepare hydroxyurea-containing agar plates, we reasoned that the toxicity observed previously on solid media might be explained by the breakdown of hydroxyurea into these toxic byproducts that are known to damage DNA. To test this idea directly, we incubated hydroxyurea at 37ºC for 48 hours and then examined the survival of wild-type and *recA* cultures treated with this agent in liquid cultures. To this end, wild-type and *recA* cultures grown to early exponential phase (OD_{600} = 0.3) were divided and treated with either 100 mM fresh or 2-day heat-decayed preparations of hydroxyurea. The viability of each culture was then followed over time. Whereas, wild-type cultures maintained viability under both conditions, *recA* cultures lost viability when treated with the 2-day old preparation of hydroxyurea. *recA* cultures were not sensitive to the freshly prepared hydroxyurea (Figure 5B).

We then examined the effect that the 2-day heat-decayed preparation of hydroxyurea had on replication. In contrast to the fresh preparation, the 2-day old preparation of hydroxyurea significantly impaired the ability of both wild-type and *recA* mutants to restore DNA synthesis following exposure (Figure 5C). The observations argue that the primary cause of hydroxyurea toxicity is direct induction of DNA damage by toxic breakdown products of hydroxyurea that accumulate over time. Consistent with this interpretation, processing of hydroxyurea by the endogenous catalases (*katE* and *katG*) of *E. coli* produces the nitric oxide intermediates responsible for hydroxyurea toxicity in plates, similar to what is observed *in vitro* and in *Arabidopsis* (51, 52). Inactivation of both classes of catalases, prevents this processing and efficiently suppresses the hypersensitivity of *recA* mutants to hydroxyurea in plates (Figure S2A). Importantly however, the time of replication recovery in the presence of fresh hydroxyurea is unaffected by the absence of catalases, even in a *recA* mutant (Figure S2B). The results strongly argue that free radical DNA damage is not associated with the observed transient inhibition of replication after hydroxyurea. To determine whether the restoration of DNA synthesis requires transcription or protein synthesis, we examined whether the recovery would occur in the presence of either rifampicin or chloramphenicol, which inhibit transcription and translation, respectively. As shown in Figure S3, DNA synthesis appears to begin to recover in the absence of either transcription or new protein synthesis. However, since new rounds of DNA replication from *oriC* also require transcription and translation, the rate of DNA synthesis declines rapidly in both mock-treated samples in the presence of either rifampicin or chloramphenicol. Thus, we cannot rule out the possibility that new protein synthesis is not required to observe a complete recovery of DNA synthesis in the presence of hydroxyurea.

Finally, we examined the survival of *nrdEF*, *nrdG*, *polB dinB umuDC*, *rnhA*, *rnhB* mutants when exposed to hydroxyurea on solid media to determine if these genes products contributed to

299 survival and were needed for either the recovery of replication or repair of hydroxyurea-induced
300 lesions. To this end, we propagated wild-type, *nrdEF*, *nrdG*, *polB dinB umuDC*, *rnhA*, *rnhB* and *recA*
301 cells in minimal medium to early exponential phase (OD_{600} = 0.3), then serially diluted and plated the
302 cultures on solid medium containing increasing concentrations of hydroxyurea. As shown in Figure 5D,
303 whereas *recA* exhibited an extreme hypersensitivity to hydroxyurea under these conditions, none of the
304 other mutants were hypersensitive. Taken together, the results indicate that the cryptic Class I
305 ribonucleotide reductase (NrdEF), Class III ribonucleotide reductase (NrdDG), the translesion DNA
306 polymerases, RNase HI (*rnhA*), or RNase HII (*rnhB*) are not required for the recovery of replication or
307 the repair of hydroxyurea-induced DNA damage.
308
309 **DISCUSSION**
310 Hydroxyurea treatment has been proposed to inhibit DNA replication through the targeted
311 inhibition of ribonucleotide reductase, eventually leading to cell death. The results presented here,
312 show that when cultures are treated with high concentrations of hydroxyurea, DNA synthesis is only
313 transiently inhibited and no loss of viability is associated with this inhibition. The recovery of
314 replication occurs even in the absence of RecA, arguing that the initial inhibition caused by
315 hydroxyurea pauses, rather than disrupts the replication machinery at the fork. Following the brief
316 period of inhibition, replication resumes and continues, even in the presence of hydroxyurea.
317 We tested the idea that reduction of rNTPs to dNTPs by *E. coli*'s two alternative ribonucleotide
318 reductases might account for the resumption of DNA synthesis in the presence of hydroxyurea. Both
319 the Class I and Class III ribonucleotide reductases are transcriptionally upregulated in response to this
320 drug (33), making this a reasonable hypothesis. However, we found no evidence for this in our study.
321 Mutants of *nrdEF* and *nrdG* restore DNA replication rates at the same time and with similar kinetics as
322 wild-type cells after hydroxyurea treatment. The restoration of DNA replication also did not depend on
323 the translesion DNA polymerases. In the presence of hydroxyurea, *polB dinB umuDC* mutants recover
324 DNA synthesis rates as well as wild-type parent. Yeast DNA polymerase ζ and *E. coli* Polymerase V
325 have been shown to alter mutagenic frequencies following hydroxyurea treatment (34, 53). However, in
326 light of the results presented here, this effect seems likely to be due to DNA damage formed by
327 byproducts of hydroxyurea, rather than an effect associated with restoring synthesis at paused forks.
328 The data we present demonstrates that extensive incorporation of rNTPs is not responsible for
329 the recovery of DNA synthesis observed in the presence of hydroxyurea. Although elevated levels of
330 ribonucleotides were detected in mutants lacking RNase HII, the frequency of incorporation was well
331 below that which could account for the observed synthesis. In fact, no difference in the rate was

observed when samples were first treated with alkali to remove ribonucleotides before DNA synthesis was quantified. It is worth noting that rNTP-dNTP pool imbalances induced by hydroxyurea have been shown to increase rNTP misincorporation frequencies *in vitro* (38, 39). Our results demonstrate that this also occurs *in vivo*. Taken together, these observations indicate that, in *E. coli*, one cannot assume that hydroxyurea treatment will result in a prolonged block to replication, even when used at high concentrations.

The results also highlight distinct differences between the mechanisms of lethality operating under conditions of hydroxyurea treatment and thymine starvation, an alternative chemotherapeutic approach associated with nucleotide depletion (54, 55). Although these two mechanisms are often compared, the data presented here and in recent studies argues strongly that hydroxyurea toxicity is mediated through DNA damage (13, 15, 33, 50–52), rather than depletion or unbalanced pools of deoxyribonucleotides (24). By contrast, thymine starvation creates clear replication abnormalities at the replication origin and terminus, suggesting unbalanced replication (56, 57), rather than DNA damage as the cause of toxicity.

Although DNA synthesis continues for several hours in the presence of hydroxyurea, the rate of replication undergoes a rapid drop immediately following hydroxyurea addition. This inhibition is only observed when extremely high concentrations (0.1M) of hydroxyurea are used, and even then, the rate of replication is restored to near normal levels within the 60-minute recovery time of the experiments performed in this study. Considering the rapid and transient nature of this inhibition, and the high concentrations required to induce the inhibition, it seems unlikely that the target of hydroxyurea is specific to ribonucleotide reductase. Rather, these effects would be more consistent with the general depletion of Fe-S centers in enzymes required for metabolism. Hydroxyurea has been shown to alter the Fe-S centers of enzyme cofactors involved in yeast redox reactions (13), target several metalloproteins in higher eukaryotes (reviewed in (11)) and inhibit PriL, a Fe-S containing replication enzyme, in *Sulfolobus* (12), suggesting a more generalized effect of this drug on proteins requiring catalytic metals, of which NrdAB is one. In addition, hydroxyurea is capable of forming complexes with iron and copper *in vitro,* potentially acting as a weak chelator (29, 30). Iron is required in a variety of biological processes and it is possible that partial chelation of these metal ions by hydroxyurea may trigger a switch in cellular metabolism. Intracellular iron levels in *E. coli* are transcriptionally regulated by Fur repression, which uses Fe^{2+} as a co-repressor (58). When iron levels are low, Fur modulates expression of iron metabolism genes involved in iron transport and storage, decreases expression of iron-containing respiratory proteins such as cytochrome oxidases (*cyoA-E*; *cydAB*), and also appears to affect expression of genes involved in diverse cellular processes like glycolysis, purine metabolism and

redox stress (58). Interestingly, *E. coli* treated with hydroxyurea upregulate expression of iron transport genes and downregulate cytochrome b (*cybB*) (33) much like what is seen under low iron growth conditions, supporting the idea that hydroxyurea induces an iron-specific cellular response. In this context, the results of our study would suggest that the transient decline and quick recovery of replication rates following hydroxyurea treatment is a product of a global sensing mechanism that *E. coli* uses for metal ion homeostasis and could explain the lack of phenotype for any of the mutants we examined. Although hydroxyurea is affecting iron regulation, we did not see evidence of reactive oxygen species or Fenton chemistry inducing DNA damage during this initial period of inhibition and recovery (13, 15, 33, 50), as *recA* mutants, which fail to replicate in the presence of DNA damage, replicated and survived as well as wild-type cells when treated with hydroxyurea.

In contrast, if hydroxyurea was stored under conditions that affected its stability (15, 32), then the recovery of replication and viability became dependent on *recA*. The behavior of replication in *recA* mutants treated with the breakdown products of hydroxyurea was indistinguishable from that observed following UV irradiation, consistent with the presence of DNA damage. These observations are consistent with work by Kuong and Kuzminov who showed that the effect of "aged" hydroxyurea on cell survival was more severe than for freshly prepared hydroxyurea, and correlated with the accumulation of hydrogen cyanide, nitric oxide and hydrogen peroxide (15). The DNA damage induced when using heat-exposed or old stocks of hydroxyurea may also resolve why hydroxyurea is so much more potent as a toxin when cells are treated with hydroxyurea that has been incorporated into agar plates as compared with direct addition of this drug to liquid cultures. Hydroxyurea incorporated into plates is at least two days old and has been exposed to temperatures above 50°C, allowing these breakdown products known to damage DNA to accumulate. These differing conditions are also likely to explain discrepancies in previous studies with respect to the hypersensitive phenotype of various repair mutants (15, 36, 59, 60).

We believe these results are most consistent with the idea that hydroxyurea added to growing *E. coli* cultures transiently disrupts metabolism by inactivating enzymes requiring transition metal chemistry (Figure 6). These include the primary ribonucleotide reductase NrdAB, as well as other targets that are required to maintain ongoing DNA synthesis, transcription, translation, and cell growth (12, 13, 58, 61). Once the iron balance is restored or compensated for, replication can continue for several hours. Toxicity from hydroxyurea is only observed after several hours and is likely to be due to DNA damage induced by toxic intermediates that have been reported to accumulate as hydroxyurea breaks down.

MATERIALS AND METHODS

Bacterial strains. Strains used in this study are summarized in Table 1. All strains are derived from SR108, a *thyA36 deoC2* derivative of W3110 (62). HL921 (SR108 Δ(*srl-recA*)*306*::Tn*10*), CL646 (SR108 *polB*::Ω Sm-Sp *dinB*::Kan^r *umuDC595*::*cat*) and CL854 (SR108 *uvrA*::Tn*10 recA*::*cat*) have been previously described (44, 63, 64). CL2602 (SR108 *nrdG*::Kan^r) was constructed by P1 transduction of the *nrdG784*::Kan^r allele from JW4196-3 into SR108 (65). CL3360 (SR108 *rnhB782*::Kan^r) was constructed by P1 transduction of the *rnhB*::Kan^r allele from JW0178 into SR108 (65). CL3362 (SR108 *rnhA*::Kan^r) was constructed by P1 transduction of the *rnhA733*::Kan^r allele from JW0204 into SR108 (65).

CL2164 (DY329 *nrdEF*::*cat*) was constructed by gene replacement using the recombineering strain DY329 (66). The cat cassette was amplified from CL646 using the nrdE:camF primer 5' CTCATGGGTACGCAAAGCGATATCGAAAACGTTCGTAAAGTGTGACGGAAGATCACTTCG and the nrdF:camR primer 5' GCGTGATAAAAAGCTATTTGGCGGGAATTATTTCCCTGCTGACCAGCAATAGACATAAGCG. The PCR product was transformed into DY329 to generate CL2164, selecting for chloramphenicol resistance. The gene replacement was then moved into SR108 by standard P1 transduction, generating strain CL2581 (SR108 *nrdEF*::*cat*).

CL1406 (DY329 *katE*::*FRT-cat-FRT*) was constructed by gene replacement using the recombineering strain DY329. The cat cassette was amplified from pKD3 (67) using the katEF-FRTCamF primer 5' TTCAGTAATAAATTAAGGAGACGAGTTCAATGTCGCAACAGGTGTAGGCTGGAGCTGCTTC and the katER-FRTCamR primer 5' GGCGCAATTGCGCCGCCTCCCATCAGGCAGGAATTTTGTCGCATATGAATATCCTCCTTA. The PCR product was transformed into DY329 to generate CL1406, selecting for chloramphenicol resistance. The gene replacement was then moved into SR108 by standard P1 transduction, generating strain CL1420 (SR108 *katE*::*FRT-cat-FRT*). The chloramphenicol resistance cassette was then eliminated from CL1420 using the temperature-sensitive plasmid pCP20 (68) encoding FLP recombinase to generate CL1424 (SR108 *katE*::*FRT*).

CL1408 (DY329 *katG*::*FRT-cat-FRT*) was constructed by gene replacement using the recombineering strain DY329. The cat cassette was amplified from pKD3 using the katGF-FRTCamF primer 5'

ACGGTAACACTGTAGAGGGGAGCACATTGATGAGCACGTCGGTGTAGGCTGGAGCTGCTTC and the katGR-FRTCamR primer 5' GCTGAACGGGGTCAGATTACAGCAGGTCGAAACGGTCGAGGCATATGAATATCCTCCTTA. The PCR product was transformed into DY329 to generate CL1408, selecting for chloramphenicol resistance. The gene replacement was then moved into CL1424 by standard P1 transduction, generating strain CL1429 (SR108 *katE*::*FRT katG*::*FRT-cat-FRT*). The chloramphenicol resistance cassette was then eliminated from CL1429 using the temperature-sensitive plasmid pCP20 encoding FLP recombinase to generate CL1438 (SR108 *katE*::*FRT katG*::*FRT*).

CL1440 (SR108 *katE*::*FRT katG*::*FRT recA*::*cat*) was constructed by P1 transduction of the *recA*::*cat* allele from CL854 into CL1438.

DNA synthesis and accumulation. UV irradiation used a 15-W germicidal lamp (254 nm) at an incident dose of 0.9 J/m^2/s. For experiments using UV irradiation, fresh overnight cultures were diluted 1:100 and grown at 37°C in Davis medium supplemented with 0.4% glucose, 0.2% Casamino Acids, 10 μg/ml thymine (DGCthy medium) and 0.1 μCi/ml [^{14}C]thymine to an optical density at 600 nm (OD_{600}) of precisely 0.3. At this time, half of the cells were mock irradiated, while the remaining culture was UV irradiated with an incident dose of 27 J/m^2.

Fresh stocks of 1 M hydrogen peroxide were prepared by diluting 50% hydrogen peroxide (14.7 M) in deionized water immediately before experimental use. For experiments using hydrogen peroxide, overnight cultures were diluted 1:100 and grown at 37°C in Luria-Bertani medium supplemented with 10 μg/ml thymine (LBthy) and 0.1 μCi/ml [^{14}C]thymine to an OD_{600} of precisely 0.3, at which point half of the cells were mock treated, while the remaining culture was treated with 10 mM hydrogen peroxide for 5 min at 37°C. Following either mock or hydrogen peroxide treatment, catalase (Fisherbrand) was added directly to the culture to a final concentration of 200 μg/ml to remove excess hydrogen peroxide from the medium.

Fresh stocks of 2 M hydroxyurea were prepared in deionized water immediately before experimental use. To prepare heat-decayed hydroxyurea, a 2 M stock of hydroxyurea in deionized water was made and stored in an air-tight tube sealed with Parafilm for 48 h at 37°C. For experiments using hydroxyurea, overnight cultures were diluted 1:100 and grown at 37°C in DGCthy supplemented with 0.1 μCi/ml [^{14}C]thymine to an OD_{600} of precisely 0.3, at which point one-fourth of the cells was mock treated, while the remaining culture was divided equally and exposed to 1 mM, 10 mM and 100 mM freshly prepared hydroxyurea to determine optimum dose. In subsequent experiments, cells were sub-cultured and grown at 37°C in DGCthy supplemented with 0.1 μCi/ml [^{14}C]thymine to an OD_{600} of

precisely 0.3, at which point half of the cells were mock treated, while the remaining culture was treated with 100 mM hydroxyurea that was freshly prepared or heat treated.

For all treatments, cultures were immediately returned to 37°C following exposure to allow continued growth and recovery in the case of UV-irradiated and hydrogen peroxide-treated cells. At the times indicated, duplicate 0.5-ml aliquots of culture were pulse-labeled with 0.5 μCi/ml [^{3}H]thymidine for 2 min at 37°C. Cells were then lysed, and the DNA was precipitated in cold 5% trichloroacetic acid and filtered onto Millipore glass fiber filters. The amounts of ^{3}H and ^{14}C on each filter were determined by scintillation counting.

To determine whether the recovery of synthesis involves extensive rNTP incorporation, cultures were grown and treated with freshly prepared 100 mM hydroxyurea as described above. At the times indicated, quadruplicate 0.5-ml aliquots of culture were pulse-labeled with 0.5 μCi/ml [^{3}H]thymidine for 2 min at 37°C. Cells from two of the four aliquots were then lysed immediately, and the DNA was precipitated in cold 5% trichloroacetic acid and filtered onto Millipore glass fiber filters as before. The remaining two aliquots were lysed and treated with alkali at a final concentration of 500 mM NaOH for 30 min at room temperature before the DNA was precipitated in cold 5% trichloroacetic acid and filtered onto Millipore glass fiber filters. The amounts of ^{3}H and ^{14}C on each filter were determined by scintillation counting.

To determine whether recovery of synthesis requires transcription or new protein synthesis, cultures were grown as described above and then divided equally into six aliquots. Three of the six aliquots were mock treated together with no inhibitor, 100 μg/ml rifampicin (transcription inhibitor) or 150 μg/ml chloramphenicol (translation inhibitor). The remaining three aliquots were treated with freshly prepared 100 mM hydroxyurea together with no inhibitor, 100 μg/ml rifampicin or 150 μg/ml chloramphenicol. Samples were collected at the times indicated, pulse-labeled with 0.5 μCi/ml [^{3}H]thymidine for 2 min at 37°C, and processed as described above.

RNA synthesis. Overnight cultures were diluted 1:100 and grown at 37°C in DGCthy to an OD_{600} of precisely 0.3, at which point half of the cells were mock treated, while the remaining culture was treated with freshly prepared 200 mM hydroxyurea. At the times indicated, duplicate 0.5-ml aliquots of culture were pulse-labeled with 0.2 μCi/ml [^{3}H]uridine for 2 min at 37°C. Cells were then lysed, and the RNA was precipitated in cold 5% trichloroacetic acid and filtered onto Millipore glass fiber filters. The amounts of ^{3}H on each filter were determined by scintillation counting.

Genomic DNA preparation and agarose gel analysis. Fresh overnight cultures were diluted 1:100 in DGCthy medium, grown at 37°C to an OD_{600} of 0.3, treated with freshly prepared 100 mM hydroxyurea and then incubated further at 37°C. At the times indicated, a 0.75-ml aliquot of culture was transferred to an equal volume of NET (100 mM NaCl, 10 mM Tris [pH 8.0], 20 mM EDTA [pH 8.0]), centrifuged for 60 sec, resuspended in 140 µl of lysis buffer (1 mg/ml lysozyme, 0.5 mg/ml RNase A in 10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0]), and incubated at 37°C for 30 min. Ten microliters of 10 mg/ml Proteinase K and 10-µl 20% Sarkosyl were then added and incubation at 37°C was performed for a further 30 min. Samples were then extracted with four volumes of phenol-chloroform (1:1), followed by four volumes of chloroform.

Samples were treated with 200 mM NaOH for 30 min and then electrophoresed on a 0.5% alkali-agarose gel in 30 mM NaOH, 1 mM EDTA at 30 V for 16 h, stained and visualized with ethidium bromide. A second set of samples was electrophoresed on a 0.5% neutral-agarose gel in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA [pH 8.0]) at 30 V for 16 h, stained and visualized with ethidium bromide.

Measuring hydroxyurea survival in liquid cultures. Fresh overnight cultures were diluted 1:100 in DGCthy medium, grown at 37°C to an OD_{600} of 0.3 and then treated with 100 mM hydroxyurea that was either freshly prepared or previously heat treated for 48 hours at 37°C as described above. At the times indicated, 0.1-ml aliquots of each culture were removed and serially diluted in DGCthy medium in 10-fold increments. Triplicate 10-µl aliquots of each dilution were then spotted on LBthy plates. Viable colonies were counted following overnight incubation at 37°C.

Measuring hydroxyurea survival on agar plates. Fresh overnight cultures were diluted 1:100 in DGCthy medium and grown at 37°C to an OD_{600} of 0.3. Triplicate 10-µl aliquots of serial 10-fold dilutions were then spotted on LBthy plates containing hydroxyurea at the indicated doses. Viable colonies were counted following overnight incubation at 37°C.

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REFERENCES

1. **Stearns, B, Losee, KA, Bernstein, J**. 1963. Hydroxyurea. A new type of potential antitumor agent. J Med Chem **6**:201.
2. **Stauber, RH, Knauer, SK, Habtemichael, N, Bier, C, Unruhe, B, Weisheit, S, Spange, S, Nonnenmacher, F, Fetz, V, Ginter, T, Reichardt, S, Liebmann, C, Schneider, G, Krämer, OH**. 2012. A combination of a ribonucleotide reductase inhibitor and histone deacetylase inhibitors downregulates EGFR and triggers BIM-dependent apoptosis in head and neck cancer. Oncotarget **3**:31–43.
3. **Piccinini, G, Foli, A, Comolli, G, Lisziewicz, J, Lori, F**. 2002. Complementary antiviral efficacy of hydroxyurea and protease inhibitors in human immunodeficiency virus-infected dendritic cells and lymphocytes. J Virol **76**:2274–2278.
4. **Segal, JB, Strouse, JJ, Beach, MC, Haywood, C, Witkop, C, Park, H, Wilson, RF, Bass, EB, Lanzkron, S**. 2008. Hydroxyurea for the treatment of sickle cell disease. Evid Rep Technol Assess (Full Rep) 1–95.
5. **Elford, HL**. 1968. Effect of hydroxyurea on ribonucleotide reductase. Biochem Biophys Res Commun **33**:129–135.
6. **Krakoff, IH, Brown, NC, Reichard, P**. 1968. Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. Cancer Res **28**:1559–1565.
7. **Young, CW, Schochetman, G, Hodas, S, Balis, ME**. 1967. Inhibition of DNA synthesis by hydroxyurea: structure-activity relationships. Cancer Res **27**:535–540.
8. **Yarbro, JW**. 1992. Mechanism of action of hydroxyurea. Semin Oncol **19**:1–10.
9. **Slater, ML**. 1973. Effect of reversible inhibition of deoxyribonucleic acid synthesis on the yeast cell cycle. J Bacteriol **113**:263–270.
10. **Sinha, NK, Snustad, DP**. 1972. Mechanism of inhibition of deoxyribonucleic acid synthesis in Escherichia coli by hydroxyurea. J Bacteriol **112**:1321–1324.
11. **Singh, A, Xu, YJ**. 2016. The Cell Killing Mechanisms of Hydroxyurea. Genes (Basel) **7**
12. **Liew, LP, Lim, ZY, Cohen, M, Kong, Z, Marjavaara, L, Chabes, A, Bell, SD**. 2016. Hydroxyurea-Mediated Cytotoxicity Without Inhibition of Ribonucleotide Reductase. Cell Rep **17**:1657–1670.
13. **Huang, ME, Facca, C, Fatmi, Z, Baïlle, D, Bénakli, S, Vernis, L**. 2016. DNA replication inhibitor hydroxyurea alters Fe-S centers by producing reactive oxygen species in vivo. Sci Rep **6**:29361.
14. **Sakano, K, Oikawa, S, Hasegawa, K, Kawanishi, S**. 2001. Hydroxyurea induces site-specific DNA damage via formation of hydrogen peroxide and nitric oxide. Jpn J Cancer Res **92**:1166–1174.
15. **Kuong, KJ, Kuzminov, A**. 2009. Cyanide, peroxide and nitric oxide formation in solutions of hydroxyurea causes cellular toxicity and may contribute to its therapeutic potency. J Mol Biol **390**:845–862.
16. **REICHARD, P**. 1962. Enzymatic synthesis of deoxyribonucleotides. I. Formation of deoxycytidine diphosphate from cytidine diphosphate with enzymes from Escherichia coli. J Biol Chem **237**:3513–3519.
17. **Turner, MK, Abrams, R, Lieberman, I**. 1966. Meso-alpha, beta-diphenylsuccinate and hydroxyurea as inhibitors of deoxycytidylate synthesis in extracts of Ehrlich ascites and L cells. J Biol Chem **241**:5777–5780.
18. **Neuhard, J**. 1967. Studies on the acid-soluble nucleotide pool in Escherichia coli. IV. Effects of hydroxyurea. Biochim Biophys Acta **145**:1–6.
19. **Rosenkranz, HS, Carr, HS**. 1966. Studies with hydroxyurea. II. Prolonged exposure of Escherichia coli to hydroxyurea. J Bacteriol **92**:178–185.
20. **Morganroth, PA, Hanawalt, PC**. 2006. Role of DNA replication and repair in thymineless death in Escherichia coli. J Bacteriol **188**:5286–5288.

21. **Skoog, L, Nordenskjöld, B**. 1971. Effects of hydroxyurea and 1-beta-D-arabinofuranosyl-cytosine on deoxyribonucleotide pools in mouse embryo cells. Eur J Biochem **19**:81–89.
22. **Skoog, L, Bjursell, G**. 1974. Nuclear and cytoplasmic pools of deoxyribonucleoside triphosphates in Chinese hamster ovary cells. J Biol Chem **249**:6434–6438.
23. **Gandhi, V, Plunkett, W, Kantarjian, H, Talpaz, M, Robertson, LE, O'Brien, S**. 1998. Cellular pharmacodynamics and plasma pharmacokinetics of parenterally infused hydroxyurea during a phase I clinical trial in chronic myelogenous leukemia. J Clin Oncol **16**:2321–2331.
24. **Koç, A, Wheeler, LJ, Mathews, CK, Merrill, GF**. 2004. Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools. J Biol Chem **279**:223–230.
25. **Stubbe, J**. 2003. Di-iron-tyrosyl radical ribonucleotide reductases. Current Opinion in Chemical Biology **7**:183–188.
26. **Martin, JE, Imlay, JA**. 2011. The alternative aerobic ribonucleotide reductase of Escherichia coli, NrdEF, is a manganese-dependent enzyme that enables cell replication during periods of iron starvation. Mol Microbiol **80**:319–334.
27. **Garriga, X, Eliasson, R, Torrents, E, Jordan, A, Barbe, J, Gibert, I, Reichard, P**. 1996. nrdD and nrdG genes are essential for strict anaerobic growth of Escherichia coli. Biochem Biophys Res Commun **229**:189–192.
28. **Nordlund, P, Reichard, P**. 2006. Ribonucleotide reductases. Annu Rev Biochem **75**:681–706.
29. **Konstantinou, E, Pashalidis, I, Kolnagou, A, Kontoghiorghes, GJ**. 2011. Interactions of hydroxycarbamide (hydroxyurea) with iron and copper: implications on toxicity and therapeutic strategies. Hemoglobin **35**:237–246.
30. **Nigovic, B, Kujundzic, N, Sankovic, K**. 2005. Electron transfer in N-hydroxyurea complexes with iron(III). Eur J Med Chem **40**:51–55.
31. **Rosenkranz, HS, Garro, AJ, Levy, JA, Carr, HS**. 1966. Studies with hydroxyurea. I. The reversible inhibition of bacterial DNA synthesis and the effect of hydroxyurea on the bactericidal action of streptomycin. Biochim Biophys Acta **114**:501–515.
32. **Jacobs, SJ, Rosenkranz, HS**. 1970. Detection of a reactive intermediate in the reaction between DNA and hydroxyurea. Cancer Res **30**:1084–1094.
33. **Davies, BW, Kohanski, MA, Simmons, LA, Winkler, JA, Collins, JJ, Walker, GC**. 2009. Hydroxyurea induces hydroxyl radical-mediated cell death in Escherichia coli. Mol Cell **36**:845–860.
34. **Godoy, VG, Jarosz, DF, Walker, FL, Simmons, LA, Walker, GC**. 2006. Y-family DNA polymerases respond to DNA damage-independent inhibition of replication fork progression. EMBO J **25**:868–879.
35. **Shimada, K, Shibata, Y, Takage, Y**. 1975. Bacteriocidal Action of Hydroxyurea on *Escherichia coli* K12 *recA*. Japan J. Microbiol. **19**:72–74.
36. **Beam, CE, Saveson, CJ, Lovett, ST**. 2002. Role for radA/sms in recombination intermediate processing in Escherichia coli. J Bacteriol **184**:6836–6844.
37. **Traut, TW**. 1994. Physiological concentrations of purines and pyrimidines. Mol Cell Biochem **140**:1–22.
38. **Nick McElhinny, SA, Watts, BE, Kumar, D, Watt, DL, Lundstrom, EB, Burgers, PM, Johansson, E, Chabes, A, Kunkel, TA**. 2010. Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases. Proc Natl Acad Sci U S A **107**:4949–4954.
39. **Yao, NY, Schroeder, JW, Yurieva, O, Simmons, LA, O'Donnell, ME**. 2013. Cost of rNTP/dNTP pool imbalance at the replication fork. Proc Natl Acad Sci U S A **110**:12942–12947.
40. **Ohtani, N, Haruki, M, Morikawa, M, Crouch, RJ, Itaya, M, Kanaya, S**. 1999. Identification of the genes encoding Mn2+-dependent RNase HII and Mg2+-dependent RNase HIII from Bacillus subtilis: classification of RNases H into three families. Biochemistry **38**:605–618.
41. **Ogawa, T, Okazaki, T**. 1984. Function of RNase H in DNA replication revealed by RNase H

defective mutants of Escherichia coli. Mol Gen Genet **193**:231–237.
42. **Barbe, J, Villaverde, A, Guerrero, R**. 1987. Induction of the SOS response by hydroxyurea in Escherichia coli K12. Mutat Res **192**:105–108.
43. **Courcelle, J, Hanawalt, PC**. 2003. RecA-dependent recovery of arrested DNA replication forks. Annu Rev Genet **37**:611–646.
44. **Courcelle, J, Carswell-Crumpton, C, Hanawalt, PC**. 1997. recF and recR are required for the resumption of replication at DNA replication forks in Escherichia coli. **Proc Natl Acad Sci U S A 94**:3714–9.
45. **Courcelle, J, Crowley, DJ, Hanawalt, PC**. 1999. Recovery of DNA replication in UV-irradiated Escherichia coli requires both excision repair and recF protein function. **J Bacteriol 181**:916–22.
46. **Courcelle, J, Hanawalt, PC**. 1999. RecQ and RecJ process blocked replication forks prior to the resumption of replication in UV-irradiated Escherichia coli. **Mol Gen Genet 262**:543–51.
47. **Courcelle, J, Donaldson, JR, Chow, KH, Courcelle, CT**. 2003. DNA damage-induced replication fork regression and processing in Escherichia coli. **Science 299**:1064–1067.
48. **Courcelle, CT, Chow, KH, Casey, A, Courcelle, J**. 2006. Nascent DNA processing by RecJ favors lesion repair over translesion synthesis at arrested replication forks in Escherichia coli. Proc Natl Acad Sci U S A **103**:9154–9159.
49. **Schalow, BJ, Courcelle, CT, Courcelle, J**. 2011. Escherichia coli Fpg glycosylase is non-rendundant and required for the rapid global repair of oxidized purine and pyrimidine damage in vivo. J Mol Biol **410**:183–193.
50. **Nakayashiki, T, Mori, H**. 2013. Genome-wide screening with hydroxyurea reveals a link between nonessential ribosomal proteins and reactive oxygen species production. J Bacteriol **195**:1226–1235.
51. **Huang, J, Kim-Shapiro, DB, King, SB**. 2004. Catalase-mediated nitric oxide formation from hydroxyurea. J Med Chem **47**:3495–3501.
52. **Juul, T, Malolepszy, A, Dybkaer, K, Kidmose, R, Rasmussen, JT, Andersen, GR, Johnsen, HE, Jørgensen, JE, Andersen, SU**. 2010. The in vivo toxicity of hydroxyurea depends on its direct target catalase. J Biol Chem **285**:21411–21415.
53. **Northam, MR, Robinson, HA, Kochenova, OV, Shcherbakova, PV**. 2010. Participation of DNA polymerase zeta in replication of undamaged DNA in Saccharomyces cerevisiae. Genetics **184**:27–42.
54. **Hanawalt, PC**. 2015. A balanced perspective on unbalanced growth and thymineless death. Front Microbiol **6**:504.
55. **Khodursky, A, Guzmán, EC, Hanawalt, PC**. 2015. Thymineless Death Lives On: New Insights into a Classic Phenomenon. Annu Rev Microbiol **69**:247–263.
56. **Sangurdekar, DP, Hamann, BL, Smirnov, D, Srienc, F, Hanawalt, PC, Khodursky, AB**. 2010. Thymineless death is associated with loss of essential genetic information from the replication origin. Mol Microbiol **75**:1455–1467.
57. **Martin, CM, Guzman, EC**. 2011. DNA replication initiation as a key element in thymineless death. DNA Repair (Amst) **10**:94–101.
58. **McHugh, JP, Rodríguez-Quinoñes, F, Abdul-Tehrani, H, Svistunenko, DA, Poole, RK, Cooper, CE, Andrews, SC**. 2003. Global iron-dependent gene regulation in Escherichia coli. A new mechanism for iron homeostasis. J Biol Chem **278**:29478–29486.
59. **Sutera, VAJ, Lovett, ST**. 2006. The role of replication initiation control in promoting survival of replication fork damage. Mol Microbiol **60**:229–239.
60. **Guarino, E, Salguero, I, Jimenez-Sanchez, A, Guzman, EC**. 2007. Double-strand break generation under deoxyribonucleotide starvation in Escherichia coli. J Bacteriol **189**:5782–5786.
61. **Py, B, Barras, F**. 2010. Building Fe-S proteins: bacterial strategies. Nat Rev Microbiol **8**:436–446.

62. **Mellon, I, Hanawalt, PC**. 1989. Induction of the Escherichia coli lactose operon selectively increases repair of its transcribed DNA strand. **Nature 342**:95–98.
63. **Courcelle, CT, Belle, JJ, Courcelle, J**. 2005. Nucleotide excision repair or polymerase V-mediated lesion bypass can act to restore UV-arrested replication forks in Escherichia coli. **J Bacteriol 187**:6953–6961.
64. **Chow, KH, Courcelle, J**. 2007. RecBCD and RecJ/RecQ initiate DNA degradation on distinct substrates in UV-irradiated Escherichia coli. Radiat Res **168**:499–506.
65. **Baba, T, Ara, T, Hasegawa, M, Takai, Y, Okumura, Y, Baba, M, Datsenko, KA, Tomita, M, Wanner, BL, Mori, H**. 2006. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol **2**:2006.0008.
66. **Yu, D, Ellis, HM, Lee, EC, Jenkins, NA, Copeland, NG, Court, DL**. 2000. An efficient recombination system for chromosome engineering in Escherichia coli. Proc Natl Acad Sci U S A **97**:5978–5983.
67. **Datsenko, KA, Wanner, BL**. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A **97**:6640–6645.
68. **Cherepanov, PP, Wackernagel, W**. 1995. Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene **158**:9–14.

Figure Legends

Figure 1 *DNA replication in wild-type cells is only transiently inhibited following chronic exposure to hydroxyurea*. [^{3}H]thymidine was added to [^{14}C]thymine-prelabeled cultures for 2 min at the indicated times following treatment at time zero. The total DNA accumulation (^{14}C) and rate of DNA synthesis (^{3}H) relative to the amount incorporated immediately prior to exposure are plotted for wild-type cells exposed to 0 mM (open circles), 1 mM (filled diamonds), 10 mM (filled triangles) or 100 mM (filled squares) hydroxyurea treatment. Graphs represent an average of at least three independent experiments. Error bars represent one standard error of the mean.

Figure 2 *The cryptic Class I and Class III ribonucleotide reductases, and translesion DNA polymerases do not contribute to the recovery of DNA replication in the presence of hydroxyurea*. [^{3}H]thymidine was added to [^{14}C]thymine-prelabeled cultures for 2 min at the indicated times following treatment with 100 mM hydroxyurea (filled symbols) or mock treatment (open symbols) at time zero. The total DNA accumulation (^{14}C; circles) and the rate of DNA synthesis (^{3}H; squares) are plotted for A) wild-type, *nrdEF* (Class I ribonucleotide reductase), or *nrdG* (Class III ribonucleotide reductase-activating enzyme) and B) *polB dinB umuDC* cells. Each graph represents an average of at least two independent experiments. Error bars represent one standard error of the mean.

Figure 3 *rNTP misincorporation does not account for replication recovery in the presence of hydroxyurea.* A) Data were obtained and plotted as in Figure 2. The total DNA accumulation (14C) in

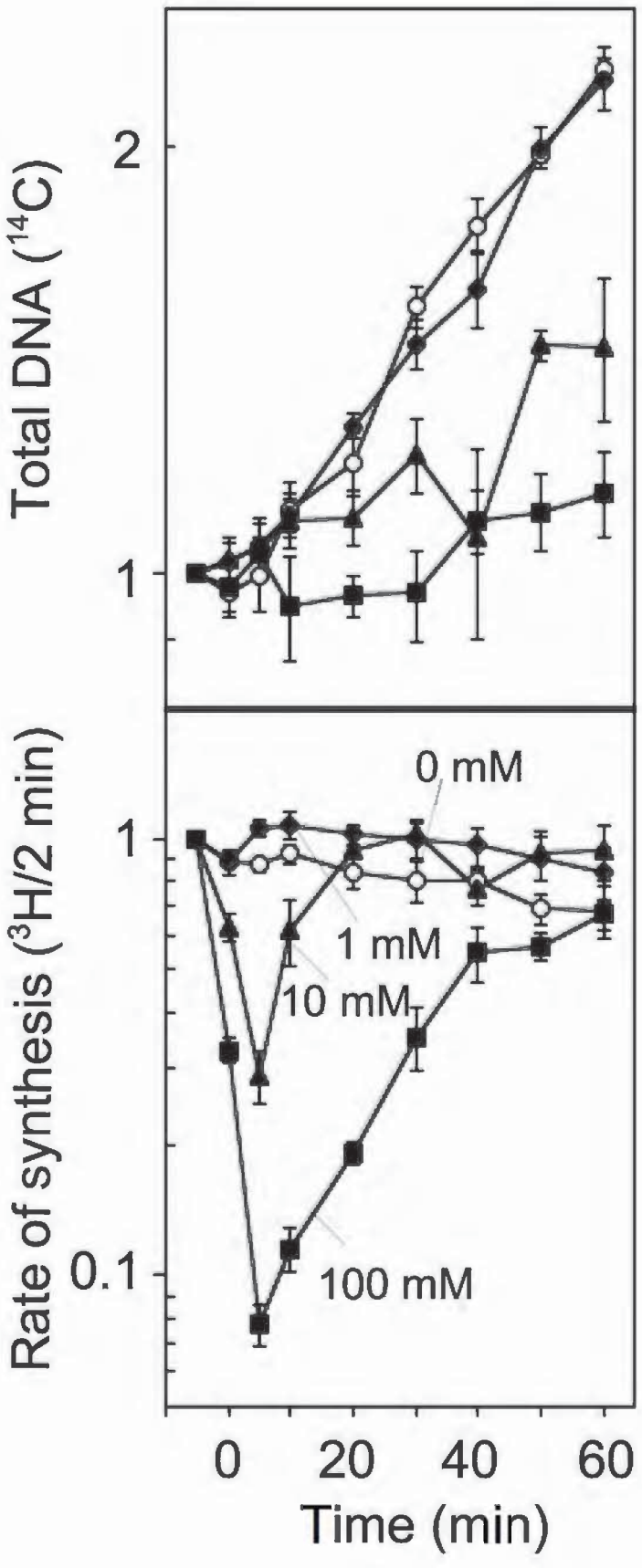
mock- (open circles) and hydroxyurea-treated cultures (filled circles); and rate of DNA synthesis (3H) in mock- (open squares) and hydroxyurea-treated cultures (filled squares) are shown for wild-type, *rnhB* and *rnhA* cells. Wild-type plot is reproduced from Figure 2. B) rNTP misincorporation during replication in the presence of hydroxyurea detectably increases in *rnhB* mutants. Wild-type, *rnhA* and *rnhB* cells were exposed to 100 mM hydroxyurea and allowed to grow at 37°C. At the indicated times, genomic DNA was purified and analyzed on alkali- and neutral-agarose gels. Representative gels are shown. C) [3H]thymidine was added to [14C]thymine-prelabeled wild-type cultures for 2 min at the indicated times following treatment with 100 mM hydroxyurea (filled symbols) or mock treatment (open symbols) at time zero. Samples were then lysed in the presence or absence of 500 mM NaOH. The total DNA accumulation (14C; circles) and rate of DNA synthesis (3H; squares) are plotted. Graph represent an average of at least two independent experiments. Error bars represent one standard error of the mean.

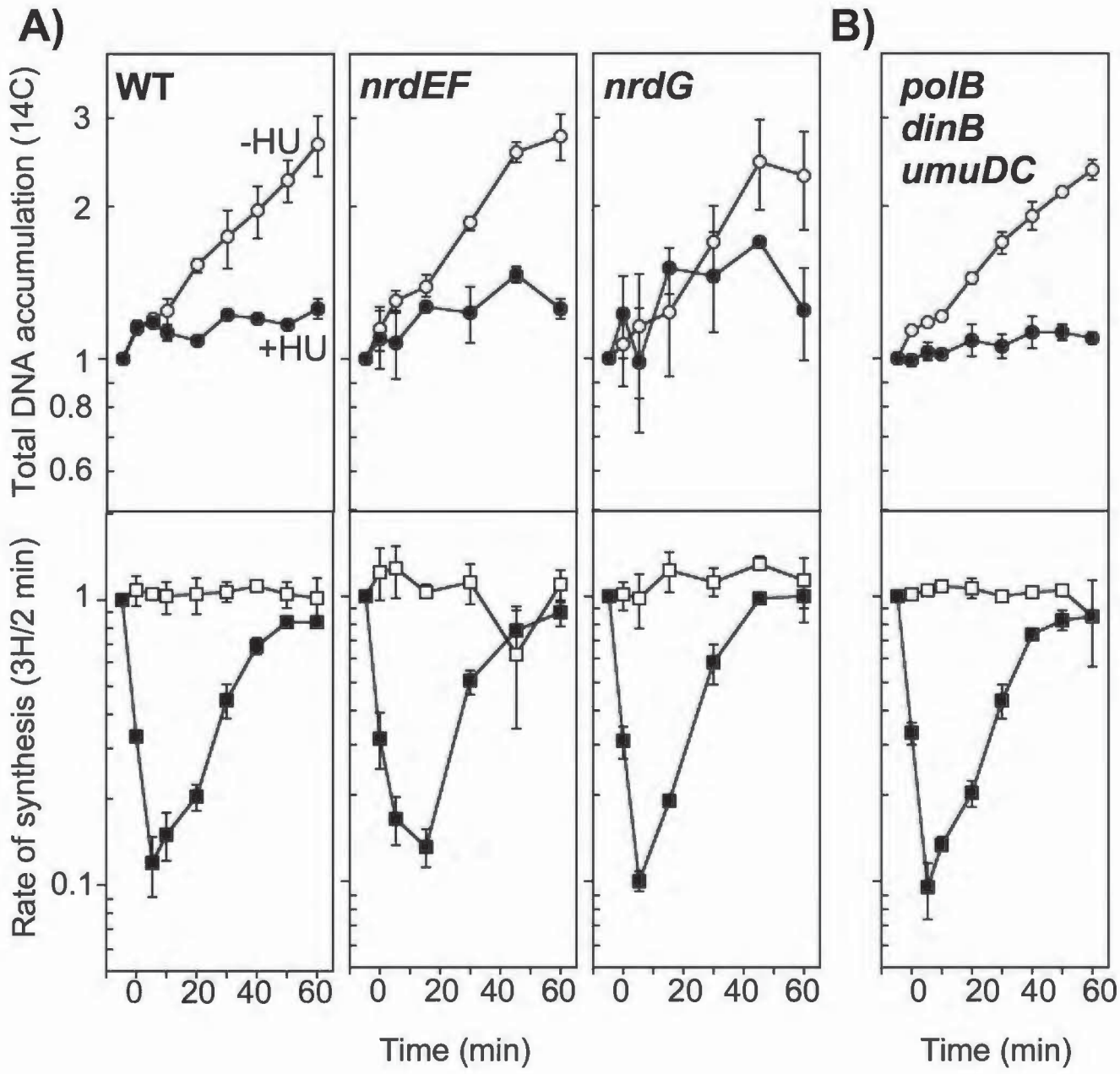
Figure 4 *RecA is not required for replication recovery following treatment with hydroxyurea, suggesting an absence of DNA damage*. A) [^{3}H]thymidine was added to [^{14}C]thymine-prelabeled cultures for 2 min at the indicated times following 27 J/m^{2} UV irradiation (filled symbols) or mock irradiation (open symbols) at time zero. The total DNA accumulation (^{14}C; circles) and rate of DNA synthesis (^{3}H; squares) are plotted. B) Cells were either exposed to 10 mM hydrogen peroxide for 5 min (filled symbols) or mock treated (open symbols) at time zero and then allowed to recover in the presence of 200 μg/ml catalase. [^{3}H]thymidine was added to [^{14}C]thymine-prelabeled cultures for 2 min at the indicated times following treatment. The total DNA accumulation (^{14}C; circles) and rate of DNA synthesis (^{3}H; squares) are plotted. C) Data were obtained and plotted as in Figure 2. The total DNA accumulation (^{14}C) in mock- (open circles) and hydroxyurea-treated cultures (filled circles); and rate of DNA synthesis (^{3}H) in mock- (open squares) and hydroxyurea-treated cultures (filled squares) are shown. Wild-type plot is reproduced from Figure 2. D) Data were obtained and plotted as in Figure 2. The total DNA accumulation (^{14}C) in mock- (open circles) and hydroxyurea-treated cultures (filled circles); and rate of DNA synthesis (^{3}H) in mock- (open squares) and hydroxyurea-treated cultures (filled squares) are shown. All graphs represent an average of at least two independent experiments. Error bars represent one standard error of the mean.

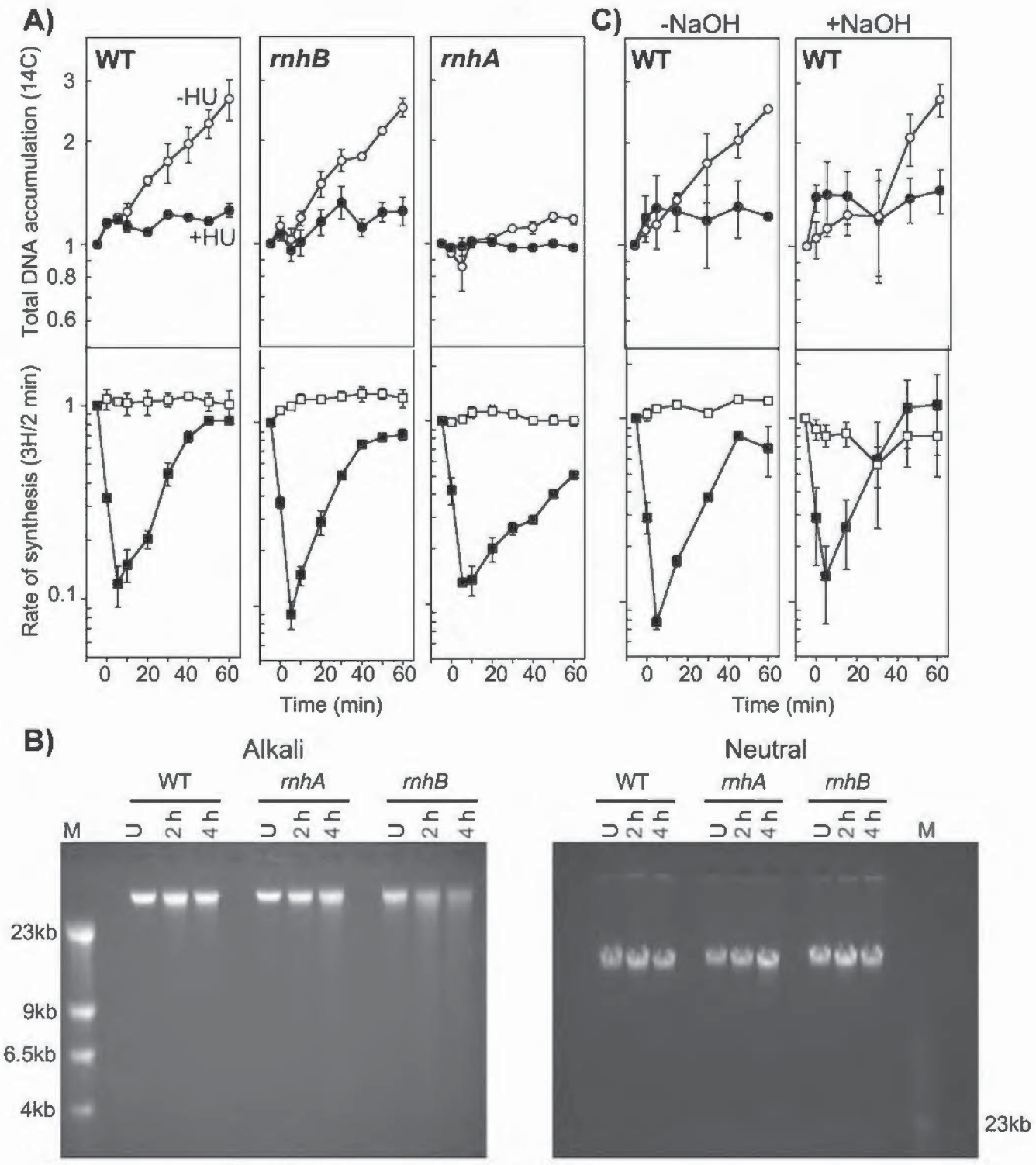
Figure 5 *RecA is required for cell survival and replication recovery following treatment with heat-decayed hydroxyurea, consistent with the induction of DNA damage by toxic byproducts of hydroxyurea.* A) Survival of wild-type and *recA* cells on agar plates supplemented with 10 mM

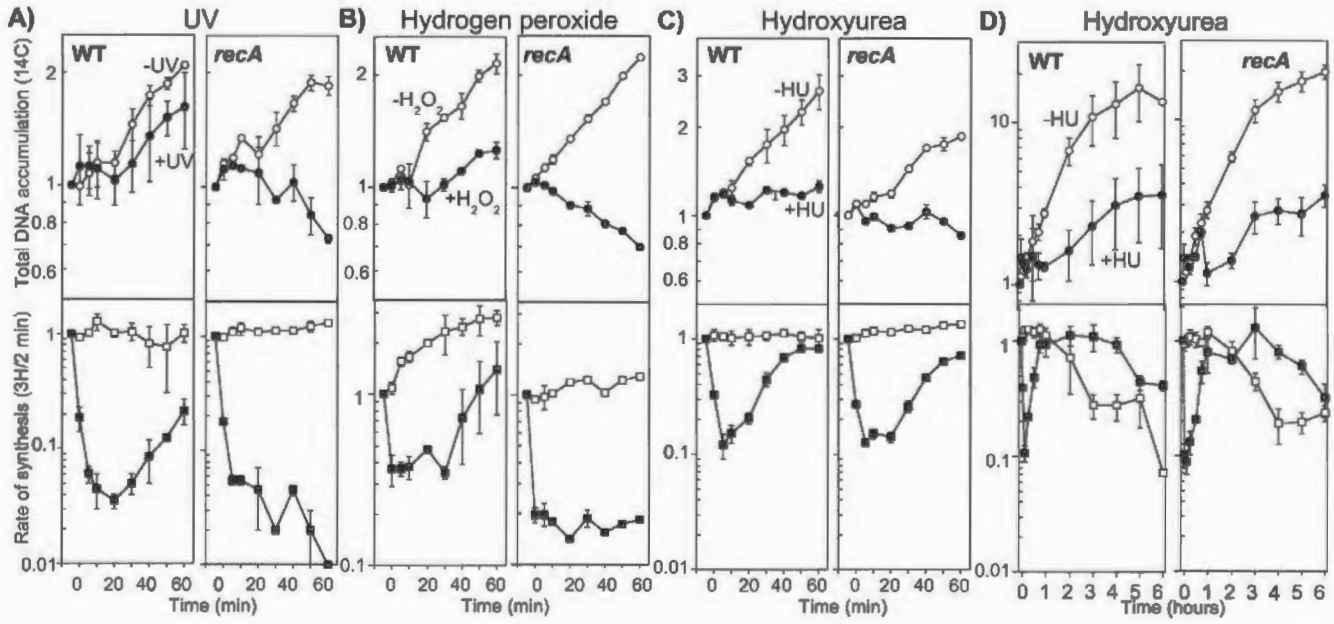
hydroxyurea. B) The survival of wild-type (squares) and *recA* (circles) cells after exposure to heat-decayed (HU+heat, closed symbols) or freshly prepared (HU, open symbols) 100 mM hydroxyurea is plotted following treatment in liquid cultures for the indicated amount of time. C) [^{3}H]thymidine was added to [^{14}C]thymine-prelabeled cultures for 2 min at the indicated times following treatment with heat-decayed 100 mM hydroxyurea (filled symbols) or mock treatment (open symbols) at time zero. The total DNA accumulation (^{14}C; circles) and rate of DNA synthesis (^{3}H; squares) are plotted. All graphs represent an average of at least two independent experiments. Error bars represent one standard error of the mean. D) RecA, but not the cryptic Class I and Class III ribonucleotide reductases, translesion DNA polymerases or ribonucleases HI and HII, is hypersensitive to hydroxyurea in plates. The survival of wild-type, parental (open squares), *nrdEF* (closed upside-down triangles), *nrdG* (closed diamonds), *polB dinB umuDC* (open circles), *rnhA* (open triangles), *rnhB* (closed triangles) and *recA* (closed circles) cultures is plotted following growth on hydroxyurea-containing agar plates at the indicated concentrations. Graphs represent an average of at least two independent experiments. Error bars represent one standard error of the mean.

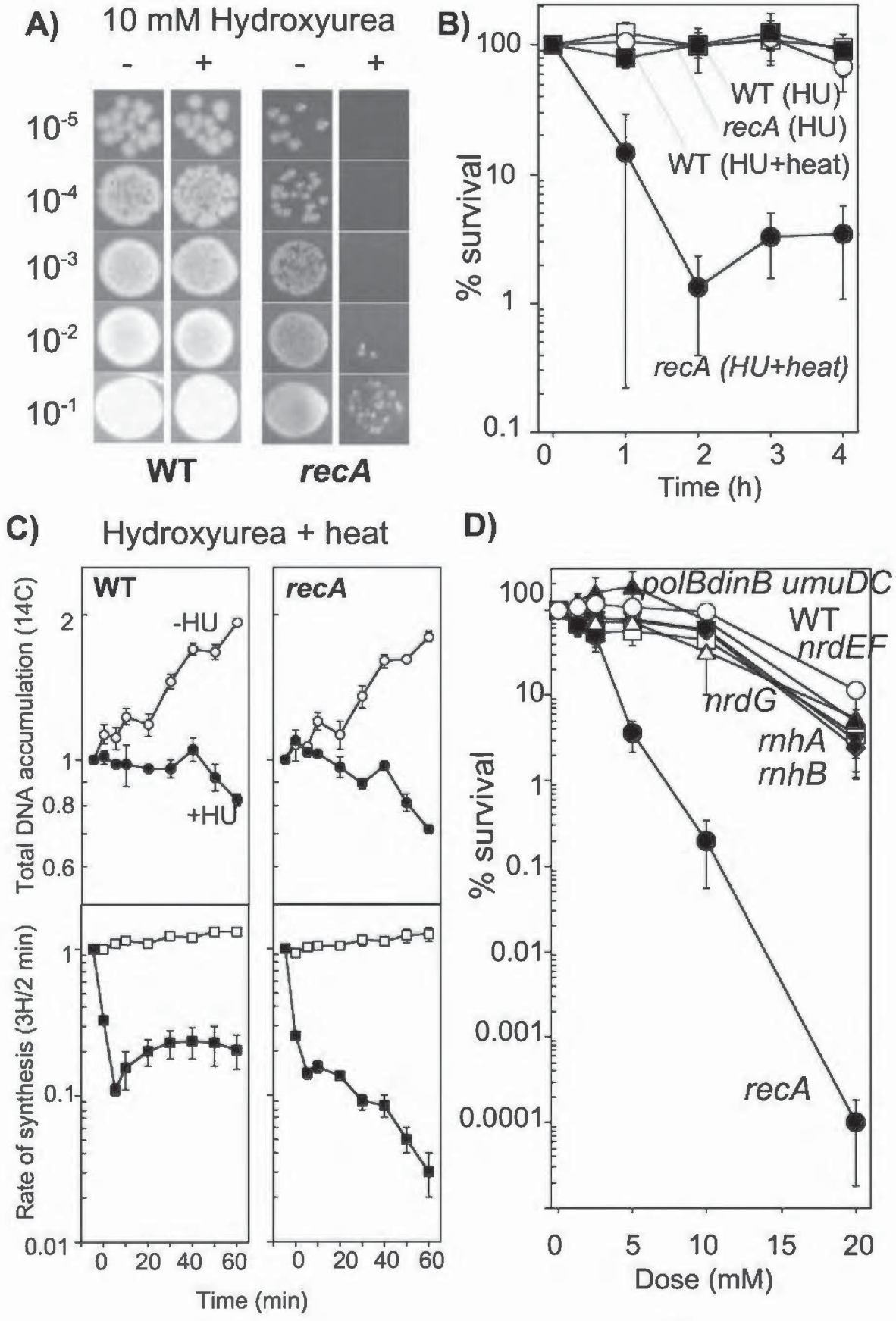
Figure 6. Model depicting the proposed effects of hydroxyurea, or heat-degraded hydroxyurea, on cellular metabolism.


Total DNA (^{14}C)
2
1
Rate of synthesis (^{3}H/2 min)
1
0.1
0 mM
1 mM
10 mM
100 mM
0
20
40
60
Time (min)


A)
B)
WT
nrdEF
nrdG
polB
dinB
umuDC
-HU
+HU
Total DNA accumulation (14C)
Rate of synthesis (3H/2 min)
3
2
1
0.8
0.6
1
0.1
0
20
40
60
Time (min)


A)
WT
rnhB
rnhA
-HU
+HU
Total DNA accumulation (14C)
Rate of synthesis (3H/2 min)
Time (min)
C)
-NaOH
+NaOH
WT
WT
Time (min)
B)
Alkali
Neutral
WT
rnhA
rnhB
U
2 h
4 h
M
23kb
9kb
6.5kb
4kb
23kb


A)
UV
B)
Hydrogen peroxide
C)
Hydroxyurea
D)
Hydroxyurea
WT
recA
-UV
+UV
-H2O2
+H2O2
-HU
+HU
Total DNA accumulation (14C)
Rate of synthesis (3H/2 min)
Time (min)
Time (hours)


A)
10 mM Hydroxyurea
-
+
-
+
10-5
10-4
10-3
10-2
10-1
WT
recA
B)
100
10
1
0.1
% survival
WT (HU)
recA (HU)
WT (HU+heat)
recA (HU+heat)
0
1
2
3
4
Time (h)
C)
Hydroxyurea + heat
WT
recA
-HU
+HU
Total DNA accumulation (14C)
2
1
0.8
0.6
Rate of synthesis (3H/2 min)
1
0.1
0.01
0
20
40
60
Time (min)
D)
polBdinB umuDC
WT
nrdEF
nrdG
rnhA
rnhB
recA
100
10
1
0.1
0.01
0.001
0.0001
% survival
0
5
10
15
20
Dose (mM)

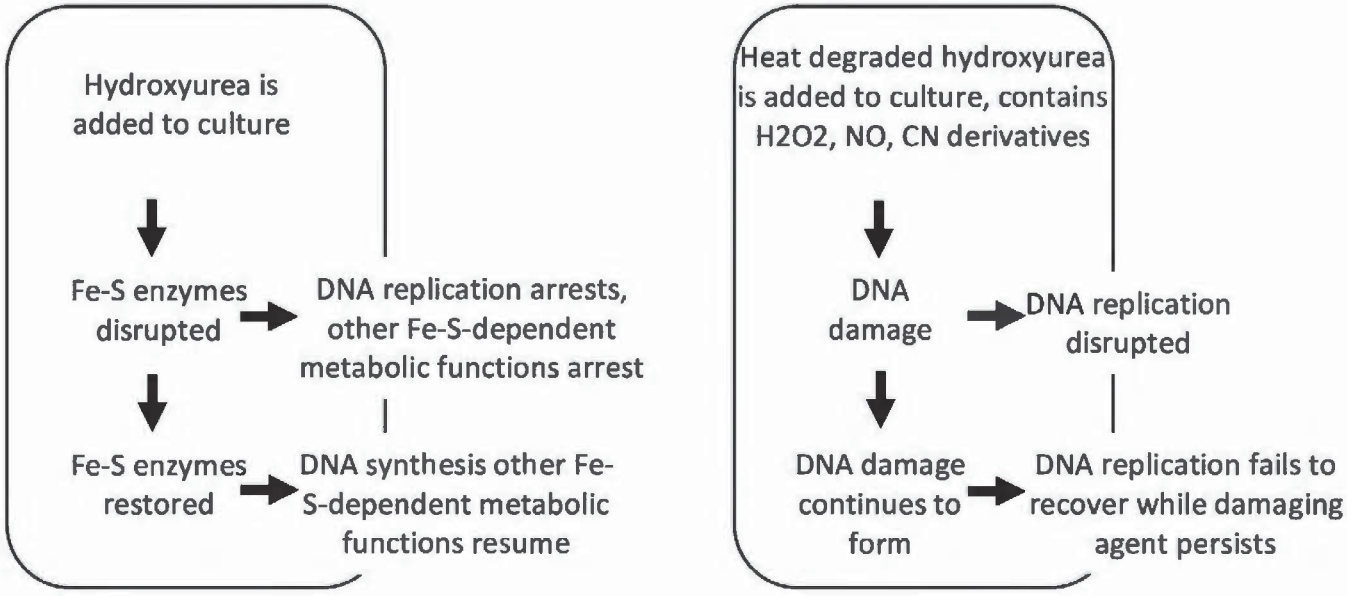

Hydroxyurea is added to culture
Fe-S enzymes disrupted
DNA replication arrests, other Fe-S-dependent metabolic functions arrest
Fe-S enzymes restored
DNA synthesis other Fe-S-dependent metabolic functions resume
Heat degraded hydroxyurea is added to culture, contains H2O2, NO, CN derivatives
DNA damage
DNA replication disrupted
DNA damage continues to form
DNA replication fails to recover while damaging agent persists

TABLE 1 E. coli K-12 strains used

Strain	Relevant Genotype	Reference or Construction
SR108	λ⁻, *thyA36*, *deoC2*, *IN*(*rrnD-rrnE*)1, *rph*	(62)
DY329	*ΔlacU169, nadA*::*Tn10*, *gal490*, λ*cI857*, Δ(*cro-bioA*)	(66)
JW4196-3	*nrdG784*::Kan^r	(65)
JW0178	*rnhB782::Kan*^{r}	(65)
JW0204	*rnhA733*::Kan^r	(65)
CL2164	*ΔlacU169, nadA::Tn10, gal490, λcI857, Δ(cro-bioA), nrdEF::cat*	DY329 x PCR fragment (nrdE-cat and nrdF-cat primers)
CL1406	*ΔlacU169, nadA::Tn10, gal490, λcI857, Δ(cro-bioA), katE*::*FRT-cat-FRT*	DY329 x PCR fragment (katEF-FRTCamF and katER-FRTCamR primers)
CL1408	*ΔlacU169, nadA::Tn10, gal490, λcI857, Δ(cro-bioA), katG*::*FRT-cat-FRT*	DY329 x PCR fragment (katGF-FRTCamF and katGR-FRTCamR primers)
Strains isogenic to SR108		
HL921	*recA*::Tn*10*	(44)
CL646	*polB::Ω Sm-Sp dinB::Kan*^{r} *umuDC595::cat*	(63)
CL854	*uvrA*::Tn*10 recA*::*cat*	(64)
CL2581	*nrdEF::cat*	SR108 x P1 (CL2164)
CL2602	*nrdG784*::Kan^r	SR108 x P1 (JW4196-3)
CL3360	*rnhB782::Kan*^{r}	SR108 x P1 (JW0178)
CL3362	*rnhA733*::Kan^r	SR108 x P1 (JW0204)
CL1420	*katE*::*FRT-cat-FRT*	SR108 x P1 (CL1406)
CL1424	*katE*::*FRT*	CL1420 x pCP20 (eliminate cat)
CL1429	*katE*::*FRT katG*::*FRT-cat-FRT*	CL1424 x P1 (CL1408)
CL1438	*katE*::*FRT katG*::*FRT*	CL1429 x pCP20 (eliminate cat)
CL1440	*katE*::*FRT katG*::*FRT recA*::*cat*	CL1438 x P1 (CL854)